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DOI: https://doi.org/10.1111/j.1574-6941.2000.tb00682.x

ELSEVIER

FEMS Microbiology Ecology 31 (2000) 173–182

FEMS MICROBIOLOGY ECOLOGY

www.fems-microbiology.org

Bacterial diversity and community composition in the chemocline of the meromictic alpine Lake Cadagno as revealed by 16S rDNA analysis

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Received 13 July 1999; received in revised form 29 November 1999; accepted 30 November 1999

Abstract

Using different techniques of molecular biology we investigated the bacterial diversity of the chemocline of the meromictic Lake Cadagno. Cloning of a total community 16S rDNA PCR product and subsequent screening with a combination of amplified ribosomal DNA restriction analysis and temporal temperature gradient gel electrophoresis (TTGE) analysis revealed that 30 of 47 randomly selected clones were unique. Partial sequencing and comparative analysis indicated a high bacterial diversity dominated by the γ-Proteobacteria (33.3%). Most of these rDNA clone sequences were not closely related to any 16S rDNA sequence in the database. In a second approach, the TTGE pattern from an environmental sample was compared with the migration of the cloned 16S rDNA fragments. Four clone types were identified on the environmental pattern by excising and sequencing comigrating bands, three of which were well represented in the library: two Chromatiaceae species and one sequence affiliated with the *Desulfobulbus* assemblage. Using the fluorescent in situ hybridization technique we essentially confirmed the results of the cloning experiments and the TTGE analysis.

Keywords: 16S rDNA; Amplified ribosomal DNA restriction analysis; Temporal temperature gradient gel electrophoresis; Fluorescent in situ hybridization; Bacterial diversity; Meromictic

1. Introduction

Meromictic lakes are interesting model systems for research in aquatic biology for several reasons, e.g. high physical stability of the water masses, clearly separated compartments and a relatively constant vertical stratification in bacterial populations, a compact and stable transition zone between the oxic mixolimnion and anoxic monimolimnion, and in many cases the presence of a dense microbial community at the redox transition zone [1]. Such a bloom of microorganisms is observed in the redoxcline of Lake Cadagno, a meromictic alpine lake in Switzerland [2]. At a depth of approximately 12 m, an extensive community of microorganisms, predominantly phototrophic bacteria, develops in the spring and summer months. In this zone, organisms which gain energy by anaerobic photosynthesis, aerobic and anaerobic respiration, and fermentation live at high cell densities and in close association. This makes the chemocline of meromictic lakes ideal for studies of the composition of microbial communities and the interactions within them [3,4].

In recent years, the redox transition zone of Lake Cadagno has been the subject of several limnological investigations [2,5–7]. However, relatively little attention has been paid to the species composition of the bacteria inhabiting this ecosystem, and most investigations have concentrated on the physiology of phototrophs, elemental cycles in the lake, and characterization of environmental factors. From these studies it is known that members of the purple phototrophic bacteria, presumably *Chromatium* and *Amoebobacter* species, contribute much to the biomass in the chemocline, and that sulfide oxidation is linked to sulfate reduction in a cycle in the bacterial layer. However, information about community structure has only recently been published [8,9]. A better understanding of the bacterial composition would yield insight into basic nutrient fluxes in this ecosystem. To our knowledge, this is the first investigation of the bacterial diversity of a meromictic lake by cloning-assisted sequencing.

Recently developed techniques of molecular biology

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PII: S0168-6496(99)00098-7

permit characterization of naturally occurring organisms via 16S rDNA cloning and sequencing. With the introduction of these methods, it has become possible to find many novel, as yet uncultivated organisms [10–12]. The main objective of the present work was to investigate the bacteria associated with the bacterial layer of Lake Cadagno by analysis of 16S rDNA sequences and combined application of different molecular techniques. A further aim was to compare the results obtained with different molecular techniques, i.e. cloning and sequencing, temporal temperature gradient gel electrophoresis (TTGE), and fluorescent in situ hybridization (FISH). To our knowledge, in this research project TTGE instead of denaturing gradient gel electrophoresis (DGGE) was used for the first time in environmental studies and for screening of a clone library.

2. Materials and methods

2.1. Site description and sample collection

Lake Cadagno is a small meromictic lake in the southern Swiss Alps. Its water chemistry is defined by the dolomite gypsum geology which leads to a sulfate-rich input in the deep water. The oxic mixolimnion reaches a depth of 11 m; below 11 m the concentration of hydrogen sulfide increases up to 1 mM at the sediment surface. The bacterial layer, easily detected by its high turbidity, is situated at the upper edge of the anoxic zone, which is still reached by light and where H_2S is available for anoxic photosynthesis.

Subsurface water samples were collected in sterile bottles from the depth of maximum turbidity at 11–12 m. Sampling date was 12 September 1996. The exact position of the chemocline was assessed by determining the chemical and physical parameters (temperature, pH, conductivity, oxygen, as well as turbidity) of the water column by a multisensor unit (Hydropolytester HPT, Züllig, Rheineck, Switzerland). 50 ml of water was filtered through 0.22 μm pore size filters (Durapore, Millipore, Bedford, MA, USA) using a syringe filtration device. The filters were placed in sterile 2-ml centrifuge tubes and covered with 1.5 ml lysis buffer (50 mM Tris [pH 8.0], 20 mM EDTA, 50 mM sucrose). After processing, the tubes were immediately frozen on dry ice until they were returned to the laboratory and then stored at −20°C until extraction.

2.2. Nucleic acid extraction

For total nucleic acids extraction, microorganisms were washed off the thawed filter by rinsing the membrane with the buffer. After centrifugation the cells were resuspended in 0.5 ml lysis buffer. 10 mg ml^{-1} of lysozyme was added, and then the suspension was incubated at room temperature for 10 min. After adding 1% (v/v) sodium dodecyl sulfate (SDS) and 100 μg ml^{-1} proteinase K, the mixture was incubated at 37°C for 30 min and at 55°C for 10 min. DNA was obtained from the lysates using standard phenol-chloroform extraction and ethanol precipitation procedures [13]. RNA was removed by incubating the aqueous solution with 5 U of DNase-free RNase for 15 min at 37°C. The effectiveness of the cell lysis procedure was confirmed by microscopic examination of samples taken after lysis treatment. The total amount of nucleic acids extracted from 50-ml water samples was approximately 0.5–1.0 μg DNA.

2.3. Amplification of 16S rRNA genes

Almost full-length 16S rDNA was amplified from genomic DNA by PCR with the oligonucleotide primers S-D-Bact-0008-a-S-20 (5′-AGA GTT TGA TCC TGG CTC AG-3′) and S-D-Bact-1492-a-A-19 (5′-GGT TAC CTT GTT ACG ACT T-3′). Amplification was carried out in 25-μl reactions with a Techne thermocycler (Witec AG, Luzern, Switzerland). Each reaction tube contained *Pfu* reaction buffer (Stratagene, La Jolla, CA, USA), 1.5 mM $MgCl_2$, 200 μM deoxynucleotide triphosphate, 1 U of *Pfu* DNA polymerase (Stratagene, La Jolla, CA, USA), 5 pmol of each forward and reverse primer, and 100 ng template DNA. PCR was run under the following conditions: initial denaturation at 95°C for 3 min; 25 cycles of 95°C for 1 min, 50°C for 1 min, and 72°C for 2 min; and a final extension step at 72°C for 10 min. Products were run on a agarose gel (2% agarose, 1×TBE [90 mM Tris-borate [pH 8.3] and 8 mM EDTA]) and viewed with ethidium bromide (0.5 μg ml^{-1}). Bands at the proper size range were cut out of the gel and purified (QIAquick gel extraction kit; Qiagen, Basel, Switzerland).

The cloned 16S rDNA fragments were reamplified by adding *Taq* DNA polymerase and an aliquot of 1 μl of the stored 16S rDNA clones directly to the PCR reaction mixture without a prior lysis step. The PCR amplification was carried out with a Idaho Technology Rapidcycler as recommended in the manufacturer's manual.

For TTGE analysis, a touchdown PCR was carried out with the Techne thermocycler using the Expand High Fidelity PCR System (Roche Diagnostics, Rotkreuz, Switzerland) with the following conditions. First, the samples were heated to 94°C for 5 min to denature template DNA. Subsequently, 20 cycles were performed beginning with an annealing temperature of 65°C for 30 s and lowering it 0.5°C every cycle, followed by 10 cycles with annealing at 55°C. Primer extension was carried out at 72°C for 90 s. For environmental samples the hot start technique was used, adding the enzymes after the initial denaturing step at 75°C. Negative controls showed no amplification in all experiments.

2.4. Clone library construction

The purified PCR product was phosphorylated at the

5′-end with T4 polynucleotide kinase (Roche Diagnostics, Rotkreuz, Switzerland) and used in a blunt-end ligation reaction with a *Sma*I-digested, dephosphorylated pBluescriptII KS$^+$ vector (Stratagene, La Jolla, CA, USA). The ligation products were transformed into *Escherichia coli* DH5α competent cells. Recombinant plasmid DNA was isolated from clones by a standard alkaline lysis method [13].

2.5. Amplified ribosomal DNA restriction analysis (ARDRA)

Forty-seven clones were characterized by *Hae*III/*Hin*fI restriction analysis. The cloned 16S rDNA PCR products were reamplified by PCR using the vector primers T7 and M13(-21). The nonpurified PCR products were digested with 10 U of the restriction endonucleases *Hae*III and *Hin*fI (Roche Diagnostics, Rotkreuz, Switzerland) for 1 h. Separation of the digested PCR fragments was performed by polyacrylamide gel electrophoresis (PAGE). Electrophoresis (6% polyacrylamide [acrylamide:*N,N′*-methylene bisacrylamide, 19:1], 1×Tris-borate-EDTA) was carried out for 90 min at 30 mA at 8°C. Gels were stained in ethidium bromide solution (0.5 μg ml^{-1}) and photographed under UV.

2.6. TTGE analysis

The 47 clones used for ARDRA, and the environmental samples were characterized by TTGE. The TTGE technique, similar to denaturing gradient gel electrophoresis (DGGE), allows the separation of equally sized PCR products in a polyacrylamide matrix based on their different melting behavior [14]. In contrast to DGGE where the denaturing environment is formed by a chemical gradient, the TTGE method requires a linear temperature gradient over the length of the electrophoresis run.

Plasmids were isolated by alkaline lysis and the rDNA inserts were amplified with primers S-D-Bact-0008-a-S-20-GC (5′-GC-clamp-AGA GTT TGA TCC TGG CTC AG-3′) and S-*-Univ-0536-a-A-18 (5′-GWA TTA CCG CGG CKG CTG-3′). DNA directly extracted from the water sample was amplified using the same primers. The combination of these primers amplified a 520-bp fragment of the 16S rDNA suitable for TTGE analysis. The GC-clamp [14] at the 5′-end of the forward primer prevented strand dissociation at high temperature during separation in the gel. TTGE analysis was performed with a DCode system (Bio-Rad Laboratories, Glattbrugg, Switzerland). 2.5 μl of the PCR samples was applied directly onto 6% (w/v) polyacrylamide gels (acrylamide:*N,N′*-methylene bisacrylamide, 37.5:1 [w/w]; 7 M urea; 1×TAE). The temperature range was between 54 and 64°C, temperature ramp rates between 0.6 and 3°C h^{-1}, electrophoresis time was between 3 and 15 h according to the temperature range and ramp rate, and voltage was between 90 and 150 V. After completion of electrophoresis, the gels were stained in ethidium bromide and photographed under UV transillumination.

2.7. Sequencing of cloned 16S rDNA PCR fragments and TTGE bands

Cloned PCR amplification products were bidirectionally sequenced with an automated DNA sequencer (Applied Biosystems, model 310), using the Taq DyeDeoxy terminator sequencing kit as described by the manufacturer with 500 ng of template and the primers S-D-Bact-0008-a-S-20 and S-*-Univ-0536-a-A-18. All 16S rDNA clones were sequenced on both strains.

For sequence determination of TTGE bands, small pieces were excised from the acrylamide gel and placed in sterilized vials. After 20 ml sterilized water was added, the samples were subjected to passive diffusion (12 h at 4°C). 10 μl of the supernatant was then used as template for a reamplification PCR using the same primer and reaction conditions as before. Excising was checked by rerunning an aliquot of this PCR product on the TTGE. 30–90 ng of PCR products was then subjected to sequence reactions with the primer S-D-Bact-0008-a-S-20 or S-*-Univ-0536-a-A-18 as described above.

2.8. Analysis of sequencing data

The FASTA search option for the EMBL database was used to search for closest phylogenetic neighbors. Sequences were submitted to the CHECK_CHIMERA program of the ribosomal database project (RDP) to detect possible chimeric artifacts [15,16]. Further analysis was performed using the program ARB [17]. The nearest relatives were inserted along with our cloned sequences into the ARB environment. The sequences were initially aligned by the ARB automatic aligner and then verified and corrected manually. PCR primer regions were excluded from phylogenetic analyses. To the consensus tree provided in the ARB database, the sequences were added by the maximum-parsimony approach. To avoid possible treeing artifacts caused by multiple mutational changes and/or regions that could not be unambiguously aligned, 50% conservation filters were generated using the appropriate tool of the ARB package. Only those positions which contained identical residues in at least 50% of all sequences of interest were included in the analysis. Filters were adjusted to the length of each sequence. The overall phylogenetic affinity was evaluated using a consensus filter for all bacteria. This resulted in the use of 378 aligned sequence positions for our own partial 16S rDNA sequences and 1138–1307 aligned positions for the nearly complete but differing in length 16S sequences of the nearest relatives. For accurate phylogenetic reconstruction group-specific filters were used at the subdivision, order or family level for each sequence using 411–473 aligned sequence

positions for our own sequences and 1235–1416 aligned positions for the nearest relatives.

2.9. FISH with a Chromatiaceae-specific probe

One of the filters of the sample collection (see Section 2.1) was thawed, the cells were washed off and used for FISH [18]. Cells were concentrated on 47 mm diameter polycarbonate filters (pore size 0.2 μm, type GTTP; Millipore, Volketswil, Switzerland). Filter sections were hybridized with 150 ng of CY3-labeled probe. Cells were viewed with an Olympus BX50 epifluorescence microscope (Olympus Optical, Volketswil, Switzerland). The following group-specific probes [19] were used: EUB338 (domain Bacteria), ALF1b (α-Proteobacteria), BET42a (β-Proteobacteria) and GAM42a (γ-Proteobacteria). Along with these known probes, a Chromatiaceae-specific oligonucleotide slightly modified from the primer described by Coolen and Overmann [20] was used. The sequence of the probe S-F-Chrom-986-b-A-20 is 5′-TTCCRAGGATGTCAAGGGCT-3′ (*E. coli* positions 986–1005). For this probe no formamide was added to the hybridization solution and the hybridization and wash temperatures (46 and 48°C, respectively) were identical to those for all other probes. As control for the hybridization we used *Allochromatium vinosum* (formerly *Chromatium vinosum*) and *E. coli* cultures.

2.10. Nucleotide sequence accession numbers

Nucleotide sequences have been deposited in the GenBank database under the accession numbers AF107316–AF107337 and AF109139–AF109142.

3. Results

3.1. 16S rRNA gene library

The extracted DNA from the chemocline of Lake Cadagno was suitable for direct use as PCR template; no additional step was necessary to remove substances that might inhibit PCR amplification. Since we focused on the bacterial community, we used primers designed to selectively amplify bacterial 16S rDNA fragments, allowing the cloning of approximately 96% of the 16S rRNA gene. From these amplification products, reflecting the native composition of the 16S rRNA genes, a library of 16S rDNA clones was constructed. The inserts from 59 randomly selected recombinants were amplified using vector-specific sequence primers. Forty-seven of these clones produced a single band of approximately 1500 bp (data not shown). This size corresponds to the expected size of the cloned 16S rDNA fragment.

3.2. ARDRA and TTGE analysis of the cloned 16S rDNA fragments

For screening the clone library for unique types, comparative restriction analysis of PCR products was performed using the two tetrameric restriction endonucleases *Hae*III and *Hin*fI. ARDRA of the selected colonies re-

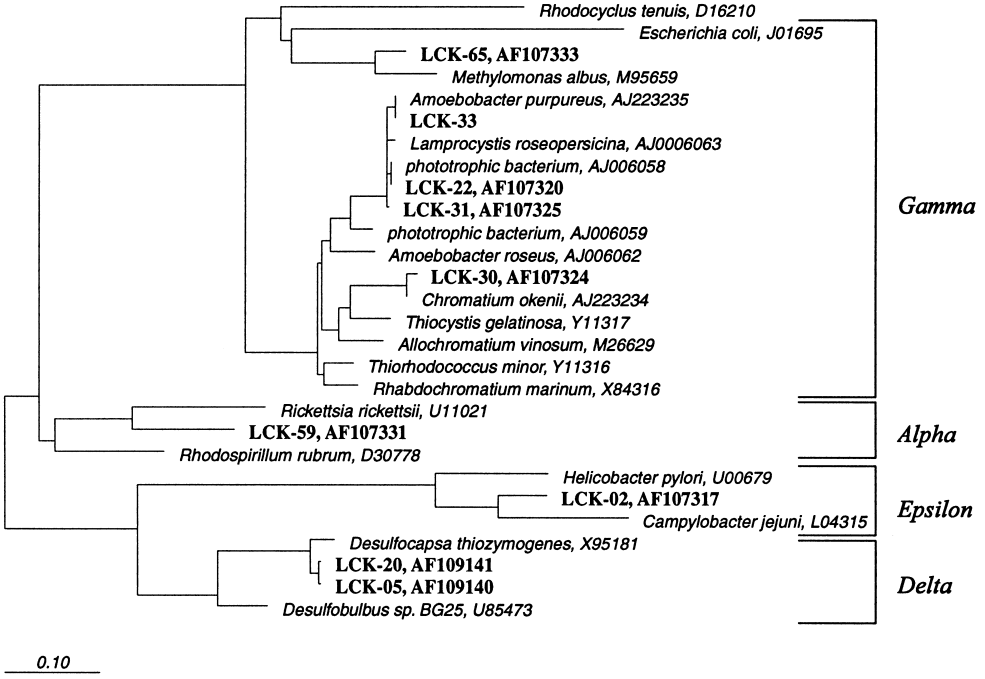

Fig. 1. Phylogenetic affiliation of the 16S rDNA clones from the chemocline of Lake Cadagno belonging to the Proteobacteria. Names are listed together with the GenBank accession numbers, cloned sequences are shown in bold. The scale bar indicates 10% estimated sequence divergence.

vealed 28 different patterns (data not shown), 22 of which were unique to single clones. One sequence type, represented by clone LCK-22, was found 11 times and accounted for 23% of all 16S rDNA clones analyzed. Besides the clones with identical ARDRA patterns, some clones seemed to share one or more fragments of similar size. Sequence comparisons showed that in all cases gene clones with similar ARDRA patterns were also phylogenetically related. To further characterize the cloned 16S rDNA fragments and to verify the results obtained by ARDRA, a TTGE analysis was performed. This technique may differentiate closely related clones which are not separated by ARDRA. Although the size of the fragments used for TTGE analysis was smaller than for the restriction analysis (approximately 520 bp instead of 1500 bp), the 11 identical clones of the ARDRA type LCK-22 were separated as three discrete TTGE patterns. Conversely, a few clones presenting identical TTGE patterns but different ARDRA patterns were also observed (four times). Sequencing confirmed that most comigrating bands were either identical in the 520-bp sequences or closely related. However, in one case two comigrating bands varied strongly in their sequence (66% similarity). Hence, TTGE does not absolutely exclude that two 16S rDNA fragments differing in their sequences migrate to the identical position in the gel. Both fingerprinting methods together showed that 30 clones (64%) were unique, indicating that the bacterial layer of Lake Cadagno is phylogenetically diverse.

3.3. Analysis of sequences cloned from environmental samples

To investigate further the relationships among the environmental clones, representatives from all ARDRA or TTGE types were partially sequenced. Approximately 520 bp were sequenced bidirectionally extending from position 8 to position 536 in reference to *E. coli* small-subunit rDNA sequence. The average length of these sequences was 473 bp, but ranged from 436 to 503 bases. Of the 30 clones sequenced, two clones were dismissed as possible chimeras.

Most sequences were not closely affiliated with presently existing sequences in public databases. Only one sequence type was identical to a known sequence obtained from the databases examined (*Amoebobacter purpureus*). The similarity values of the clones sequenced to database sequences ranged between 76% and 100%. In fact, for 46.6% of the clones maximum similarity was below 90%. The distribution of the 45 clones into the bacterial divisions is given in Table 1. We found sequences belonging to seven of the currently recognized bacterial divisions. One sequence (LCK-60) had an unusual 26-base insertion at position 84 (*E. coli* numbering), which showed no homology to any bacterial 16S rDNA. Three clones (LCK-04, LCK-41 and LCK-60) exhibited sequence similarities lower than 80% to known sequences making their phylogenetic affiliation difficult. Therefore, we did not classify these clones to a certain phylum. The two closely related clones LCK-15 and LCK-23 were most similar to an unidentified bacterium strain BD2-14 (GenBank accession number AB015542) at 87% identity. Together, these strains form a cluster which had no specific association with known divisions (similarities lower than 73%). For this reason, we did not consider the two sequences for the construction of the phylogenetic tree.

The largest number, 15 clones (33.3%), were found within the γ-Proteobacteria lineage, and 13 of these sequences grouped with the Chromatiaceae. The sequences of the two clones LCK-33 and LCK-44, representing different ARDRA types, are identical to those of the published sequence of *Amoebobacter purpureus* (AJ223235) for the 500 nucleotide positions sequenced. Most of the *Flexibacter-Cytophaga-Bacteroides*-, *Verrucomicrobia*- and green non-sulfur bacteria-like sequences showed similarities to sequences from other environmental clone libraries.

Table 1
Phylogenetic distribution of the 45 clones examined at the division level

Phylogenetic group	Number of clones	Redundant clones
α-Proteobacteria	1 (2.2%)	
γ-Proteobacteria	15 (33.3%)	LCK-22,-33,-65 (5×, 5×, 2×)
δ-Proteobacteria	5 (11.1%)	LCK-20 (4×)
ε-Proteobacteria	3 (6.7%)	LCK-02 (3×)
Proteobacteria total	**24** (53.3%)	
Cytophaga-Flexibacter-Bacteroides	8 (17.8%)	LCK-24 (3×)
Cyanobacteria	2 (4.4%)	
Green sulfur bacteria	1 (2.2%)	
Verrucomicrobia	1 (2.2%)	
High G+C Gram-positive bacteria	1 (2.2%)	
Green non-sulfur bacteria	3 (6.7%)	LCK-51 (2×)
Unclassifiable	5 (11.1%)	
Total	**45** (99.9%)	

Clone LCK-60 containing a 26-bp insertion, LCK-04, -41, -15, and -23 could not be affiliated to any division (< 80% similarity to any known division). The last column indicates the frequency of clones represented more than once in the library.

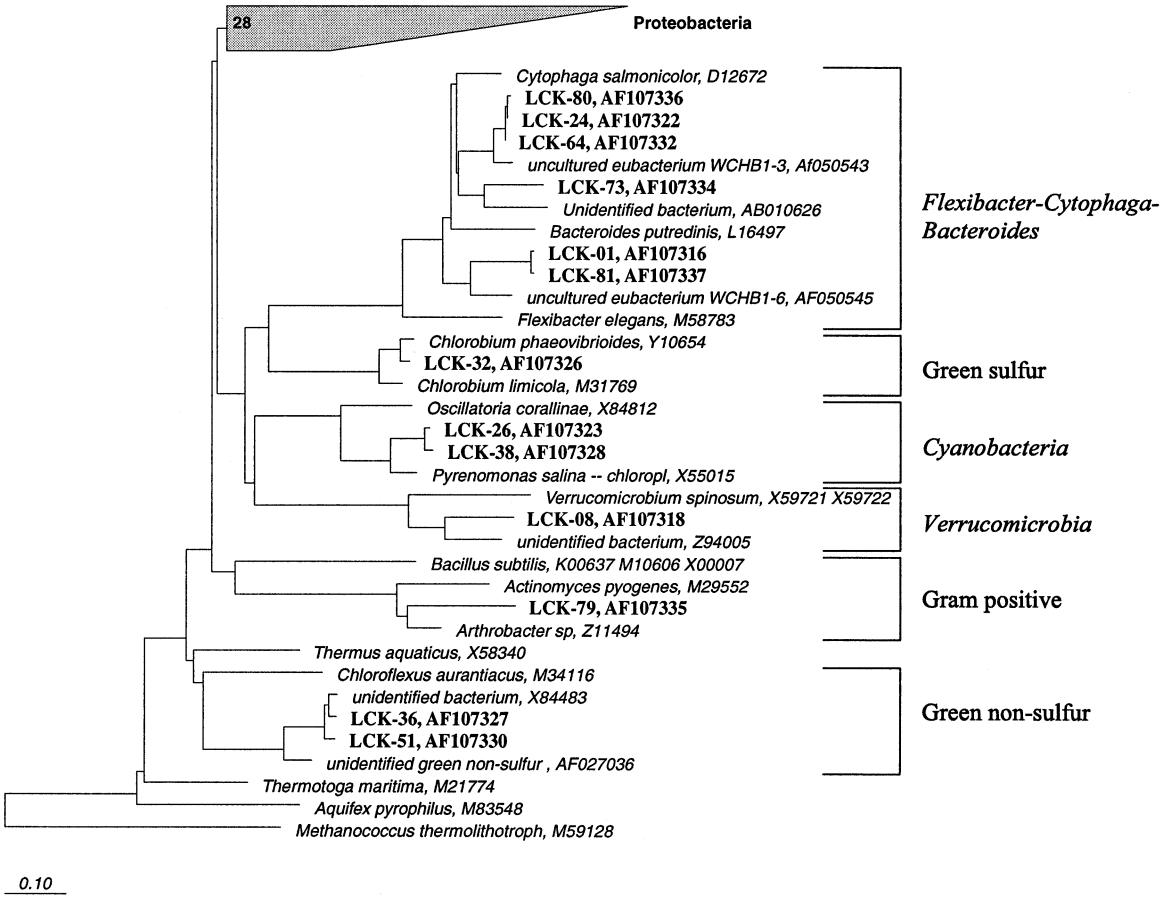

Fig. 2. Phylogenetic affiliation of 16S rDNA clones from the chemocline of Lake Cadagno. Shown is the domain Bacteria; *Methanococcus thermolithotrophicus* served as an outgroup. Names are listed together with the GenBank accession numbers, cloned sequences are shown in bold. The scale bar indicates 10% estimated sequence divergence.

The phylogenetic positions of all different clone types (except those mentioned above) are shown in Figs. 1 and 2.

3.4. TTGE analysis of the environmental samples

To determine whether the abundance of particular 16S rDNA fragments in our clone library represents the actual quantitative abundance of the sequences in the environmental sample, we applied a TTGE profiling approach. The TTGE technique is based on the same principle as DGGE, but does not require a chemical denaturing gradient [21,22]. The TTGE patterns obtained were highly reproducible. The analysis of the bacterial community in the layer by TTGE resulted in many distinguishable bands: on average at least 15 bands. It cannot be excluded that one particular band contains more than one sequence. The number of bands on a DGGE gel may not accurately reflect the number of different sequences in an environmental sample, but the most abundant species amplified should be represented in the band pattern [14]. Comparing the TTGE profile of the environmental sample with the TTGE profile of the individual clones (Fig. 3), four well represented clone types (LCK-20, 22, 24, and 33) were identified which produced bands that comigrated with intensely stained TTGE bands in the community DNA. Excision, reamplification and sequencing of these bands confirmed sequence identity to three clones. Two of these belong to the Chromatiaceae: LCK-33 is 100% identical with *Amoebobacter purpureus* and LCK-22 99.8% identical to phototrophic bacterial 16S ribosomal RNA (accession number AJ006058) and 99% to *Lamprocystis roseopersicina* (AJ006063). The third, LCK-20, is closely related to *Desulfocapsa thiozymogenes* (96.4%). The TTGE band within the sample lane which seems to align with clone LCK-24 yielded no usable sequence data. Together, these four clone types accounted for 43% of the clones investigated. Additionally, a fifth fine band in the environmental pattern was identified by sequencing as a single clone type (LCK-32) affiliated with green sulfur bacteria. The TTGE fingerprint of the chemocline was compared with fingerprints of samples collected 1 year (4 September 1997) and 2 years later (9 September 1998). The three patterns were highly similar, only band intensities varied (data not shown).

3.5. FISH of environmental samples

In the samples of the chemocline, the percentages of

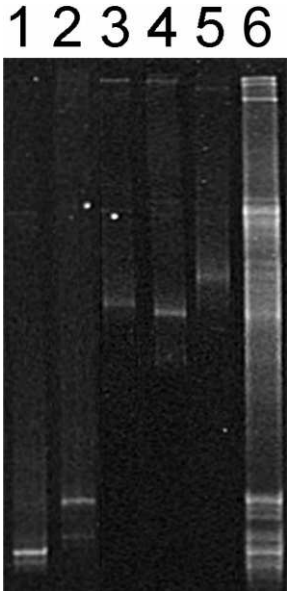

Fig. 3. Ethidium bromide-stained TTGE separation pattern of bacterial DNA fragments encoding the 16S rRNA. Lanes 1–5 show the fragments obtained from 16S rDNA clones LCK-33 (lane 1), LCK-22 (lane 2), LCK-24 (lane 3), LCK-20 (lane 4), and LCK-32 (lane 5). Lane 6 shows the band pattern of the 16S rRNA genes which were amplified directly from DNA extracted from the sample of the chemocline of Lake Cadagno.

DAPI-stained cells that could be visualized with bacterial probe EUB338 were 53–61%. 29–36% of the cells detected by DAPI hybridized with the probe for members of the gamma subclass of Proteobacteria (GAM42a), ALF1b detected 0–1% and BET42a 1–2%. Recently, Coolen and Overmann [20] stated that the region from positions 986 to 1005 of the 16S rRNA (*E. coli* numbering) is conserved for most of the bacteria belonging to the Chromatiaceae. However, applying a sequence check we found that by changing the base at position 1001 (*E. coli* numbering) from C to Y the probe recognizes additional sequences of this family without becoming more unspecific. This modification was introduced on the basis of sequence comparisons of all available Chromatiaceae sequences in current databases. We named this modified probe S-F-Chrom-986-b-A-20. The probe was tested with the two reference strains *Allochromatium vinosum* and as negative control *E. coli*. Cells of *E. coli* displayed no signals, whereas all *Allochromatium vinosum* cells were positive. Using the sample originating from the chemocline of the lake about 13–19% of the DAPI-stained cells could be assigned to the Chromatiaceae group. The results obtained with FISH correlate well with the results of the cloning and TTGE experiments.

4. Discussion

We have used the meromictic Lake Cadagno as a model system to investigate the bacterial community present at the upper edge of the anoxic zone. Investigations of the composition of the microbial community are basic for studying aspects of aquatic microbial ecology where bacteria interact with one another and with their environment. As this cannot be realized using cultivation-based methods, we used molecular approaches based on rDNA sequences to study the bacterial layer community.

To obtain an overall view of the bacterial diversity of the layer we used three different approaches: cloning-assisted sequence analysis, TTGE of amplified 16S rRNA gene fragments and FISH. Analysis of the different clones by TTGE mainly confirmed the initial data obtained by ARDRA. The TTGE method proved to be excellent in distinguishing close relatives; however, a few not identical fragments comigrated and therefore could not be resolved at the conditions applied. All 30 different ARDRA or TTGE profiles were partially sequenced. We analyzed only the first 490 bp (*E. coli* numbering) of the 16S rRNA gene including one of the most informative regions (sixth helical element, *E. coli* position 60–110) to characterize the bacterial composition. Partial sequences containing this region can be readily used to assign a sequence or an organism to the group of its closest relatives or to prove them to be new [23]. For proper phylogenetic reconstruction, longer sequences must be used. Therefore, the phylogenetic analysis undertaken with the 500-nucleotide stretch allowed sufficient assignation of the 16S rDNA clones to a main line of descent. The cluster of the two clones LCK-15 and LCK-23 with an unidentified bacterium which could not be classified stably in the known phylogenetic span of the domain Bacteria putatively forms a novel division. This finding is consistent with studies of other authors, who have routinely shown that the vast majority of the 16S rRNA genes recovered from aquatic ecosystems belong to previously uncharacterized species and, in some cases, to undescribed novel divisions of Bacteria [24–26]. Since some of these new lineages are represented by only one sequence, analysis of further sequences will be necessary to confirm their positions in the phylogenetic tree.

Based on microscopy and quantification of photosynthetic pigments previous studies on the chemocline of Lake Cadagno described the predominance of members of the Chromatiaceae, especially *Chromatium okenii* [2]. The sulfide-rich interface of Lake Cadagno is still reached by light and forms an ideal habitat for phototrophic purple bacteria. Sequence analysis showed the presence of different species of the Chromatiaceae. Interestingly, only one clone (2.2%) was closely related to *Chromatium okenii* (LCK-30; 99.4%), whereas 26.6% were identical or closely related to *Amoebobacter purpureus*. A similar dominance of the *Amoebobacter* species has also been found in other meromictic lakes [3]. However, as the sample analyzed was from late summer it cannot be excluded that the composition varies during the summer season. Besides the purple sulfur bacteria we also found members of the other photo-

trophic bacterial divisions, green sulfur and green non-sulfur bacteria. Green bacteria have not been described so far in Lake Cadagno from microscopical studies or pigment analysis [2]. Øvreas et al. [4] reported that, in the chemocline of the meromictic Lake Sælenvannet, green sulfur bacteria were dominant. In contrast, only one clone of our library could be assigned to the Chlorobiaceae group (LCK-32), while 6.7% of the clones belong to the *Chloroflexus* group which are known for photoautotrophic or photoheterotrophic growth. 11.1% of the sequences in the clone library matched with sulfate reducers of the δ-Proteobacteria. This finding is not astonishing because a sulfur cycle within the chemocline was expected from previous and ongoing [27] studies. As in other investigations on the microbial diversity we found sequences that are related to uncultured groups of bacteria like Verrucomicrobia, environmental clones within the *Cytophaga-Flexibacter-Bacteroides* group and unidentified green non-sulfur bacteria. For example, clones within the *Cytophaga* lineage grouped with clones from a contaminated aquifer [28] or clones affiliated to the green non-sulfur bacteria grouped with clones from activated sludge [29].

Although we do not know the full extent of the microbial diversity that exists in the layer, it is likely that the collection of 47 cloned 16S rDNA fragments barely represents the entire in situ diversity. The fact that only a small number of redundant clones were found is interpreted as an indication of a higher bacterial diversity. Therefore, the number of clones analyzed is insufficient with respect to the high bacterial diversity in the lake. A high microbial diversity and a limited size of analyzed clones have the danger of overestimating unique clones. Moreover, the abundance of the 16S rDNA clones in a clone library may not necessarily represent the actual abundance of the organisms found in the natural ecosystem, because clone frequencies are influenced by e.g. different numbers of RNA operons [30], uneven extraction of DNA from environmental samples [31], differences in PCR amplification of DNA from heterogeneous templates [32,33], or biases in the efficiency of cloning [34]. Therefore, it can only be assumed that multiple detection of identical ARDRA patterns within the clone library indicates a high abundance of this sequence in the original sample.

Hence, to assess the abundance of particular clones we used the TTGE analysis as a second approach to determine whether the dominant phylotypes in the clone library correspond to well-represented phylotypes on TTGE gels of community DNA. The main advantage of the TTGE technique is that it circumvents the construction and screening of clone libraries and therefore it is a convenient and efficient alternative to the often tedious and expensive cloning procedure, furthermore it prevents cloning bias. Comparing amplicons of the clone inserts next to the environmental fingerprint on TTGE gels indicated possible matches. It appears from these analyses that the two methods, cloning and TTGE, agree well, since four abundant clone types representing 43% of all clones comigrated with intense bands of the environmental sample. Since comigration does not ensure sequence identity, the TTGE bands were sequenced. Three of the excised bands showed sequence identity to the respective clones. Two of them belong to the *Amoebobacter* group (Chromatiaceae, γ-Proteobacteria) and one to the *Desulfobulbus* assemblage (δ-Proteobacteria). Both the multiple occurrence in the clone library and the intense bands on the TTGE gel argue for an important role of these organisms in the bacterial community of the chemocline. Another interesting point emerging from the sequence comparisons and the TTGE analysis is that several gene sequences are highly similar but not identical to other rDNA sequences of the clone library or to sequences in the data bank. Since some of these related sequences were also represented by different bands in environmental TTGE profiles, we do not believe that these small differences result from sequencing errors or mutations during amplification of the 16S rDNA genes. The occurrence of sets of phylogenetically related bacterial populations that coexist in the same microecosystem is observed commonly among 16S rDNA clones retrieved from environmental nucleic acids [35] and might reflect the general case. At this time, the significance of such microheterogeneity in rRNA gene clones remains unclear but it could be the result of sequence heterogeneity within a multicopy rRNA family or the result of microgeographical adaptation as a result of selection. Since we wanted to exclude the possibility that the sample of 12 September 1996 is not representative for the bacterial community in the chemocline for the time of year, two additional samples (of 1 year and 2 years later) were investigated by TTGE. The almost identical banding patterns indicate that the analyzed dominant fraction of the bacterial community seems to be characteristic for this time.

In the approach by in situ hybridization the new probe S-F-Chrom-986-b-A-20 proved to be suitable to quantify the Chromatiaceae in the environment. All three strategies (cloning, TTGE and FISH) show a dominance of Proteobacteria in the chemocline. A predominance of Proteobacteria has also been reported in a recent study of Lake Cadagno using FISH [8]; however, the quantities of the Proteobacteria differ. The reason for this incongruity is not known, but such differences in the microbial community could simply be the reflection of the changing populations present at these sites when the samples were collected. Egli et al. [36] found that the turbidity in the chemocline of Lake Cadagno often shows large and rapid temporal fluctuations at the same site indicating that the bacterial layer cannot be considered a homogeneous cell suspension. It may be that environmental changes, such as seasonal fluctuations or bioconvection processes, influence the distribution of populations within the layer.

Our analysis of the phylogenetic composition of the bacterial layer of Lake Cadagno is in line with earlier findings that natural ecosystems consist of a significant

diversity of bacterial populations and that the vast majority of these bacteria have never been described [12]. Although studies such as this one give insights into bacterial diversity, it should never be assumed that they are comprehensive because of biases in the methods. Moreover, the composition of the community is also expected to change with time. To determine the ecological importance of the analyzed organisms, further studies will be necessary. The present study indicates a highly diverse bacterial community dominated by a few species in the chemocline of Lake Cadagno and reveals hitherto not described sequences of unknown organisms. It would now be interesting to examine whether the community composition observed in Lake Cadagno could be recovered in similar habitats. This work further nicely shows the congruence and mutual supplementing of different molecular approaches to investigate microbial diversity. The authors believe that combinations of methods, e.g. ARDRA and TTGE, reveal more precise results than a single method.

Acknowledgements

We are grateful to Elke Karaus, Lucas Lüthy and Markus Fritz for technical help and Alex Widmer for his advice. This research was supported by Grant 31-50950.97 from the Swiss National Foundation for Scientific Research.

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